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Immunohistochemical Study on Epidermal Growth Factor (EGF) Receptor during Carcinogenesis in the Rat Liver

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Abstract

The expression and localization of epidermal growth factor (EGF) receptor were investigated immunohistochemically using anti-EGF receptor antibody in the normal rat liver and 3′-methyl-4-dimethylaminoazobenzen (3′-Me-DAB) induced tumors in rats.

In 8 weeks after 3′-Me-DAB treatment, multiple nodules of cholangiocarcinoma were found in the rat liver, and atypical nodules of hepatocytes were also found 14 weeks later. Immunoreactive products against EGF receptor were only slightly positive in the normal liver, the nodule of cholangiocarcinoma, and atypical nodule of hepatocytes. It was noted that EGF receptor immunoreactivity was more intense in non-cancerous tissue adjacent to tumorous nodules than in the cancerous tissue.

The present finding suggests that the expression of EGF receptor may be associated with regenerating as well as carcinogenetic processes in the rat liver.

Introduction

Epidermal growth factor (EGF) has recently been recognized as an important cell growth factor, and numerous studies on EGF and its receptor have been reported to date. In 1984, Ullrich *et al.*[1] determined amino acid sequence of EGF receptor by using cDNA clones, which revealed that EGF receptor consists of 1186 amino acids (Fig. 1)[2]. Particularly, EGF receptor has been a matter of interest, because a part of its structure closely resembles tyrosine kinase activity domain of *v-erb B* oncogene products[3-4]. In 1978, Haigler *et al.*[5] reported excessive expression of EGF receptor in human carcinoma cell line A-431. Since then, there have been many reports on excessive expression of EGF receptor in various tumors including squamous cell carcinomas of the skin, vulva, and esophagus, breast cancer, and gastric cancer[6-11].

However, information is scarce regarding immunohistochemical studies on EGF receptor.

Key words: Immunohistochemistry, Epidermal growth factor receptor, Carcinogenesis, 3′-methyl-4-dimethylaminoazobenzen, Cholangiocarcinoma, Regeneration
索引語：免疫組織化学，上皮細胞増殖因子受容体，発癌，3′-メチル-4-ジメチルアミノアゾベンゼン，胆管癌，再生
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Damjanov *et al.*[12] first demonstrated the presence of EGF receptor immunohistochemically in the cytoplasm of normal hepatocytes. In the present study, the expression and localization of EGF receptor were investigated in the liver during the process of induced carcinogenesis using immunohistochemical techniques and compared the findings with those in the normal rat liver.

Materials and Methods

1. Animals and experimental protocol

Five-week-old male *Donryu* rats (weighing ca. 200 g) were purchased from Oriental Bio Co. (Kyoto). Four control rats (normal group) were fed with regular pellet diet (Oriental Yeast) and given tap water *ad libitum*. Eight experimental rats (liver tumor group) were fed with diet containing 0.06% 3′-methyl-4-dimethylaminoazobenzen (3′-Me-DAB) *ad libitum*. Rats were given water *ad libitum* and kept in the warm room of 27°C during feeding. At 8, 10, 12, 14 weeks after the beginning of feeding, two experimental rats at each time were sacrificed by bleeding from the inferior vena cava under diethylether anesthesia. Control rats were also sacrificed at the same times as above.

Daily intake of diet per rat and body weight gain in the experimental group were less than those in the normal group.

2. Specimens of liver tissues

Liver tissues obtained from two experimental rats and one control rat were fixed in 4% paraformaldehyde for 48 hrs, embedded in paraffin, and sectioned. Five micron thick paraffin sections which were placed on albumin-coated slides were examined histopathologically after hematoxylin and eosin (H.E.) staining. The expression and localization of EGF receptor were immunohistochemically examined using anti-EGF receptor polyclonal antibody according to the methods described below.

3. Anti-EGF receptor antibody

The first antibody used in immunostaining is the rabbit anti-EGF receptor polyclonal antibody produced against synthetic peptides corresponding to the 1173rd–1186th amino acid residues of human EGF receptor, which are part of the autophosphorylation site of EGF receptor (see Fig. 1). This antibody was purified by affinity chromatography by Akiyama *et al.*[13–15].

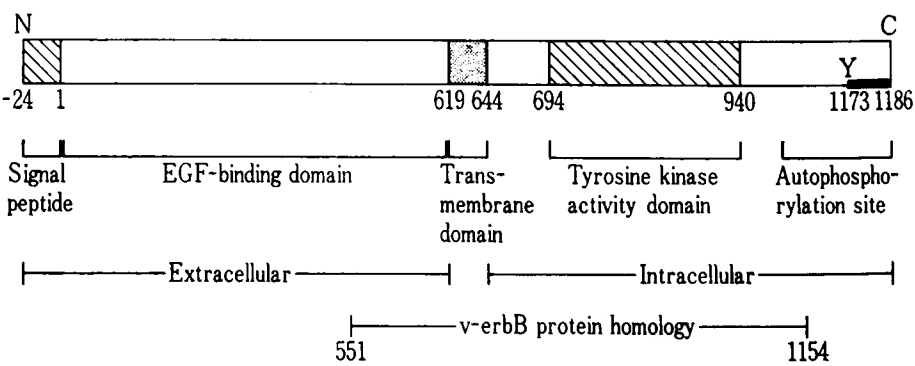

Fig. 1 Structure of EGF receptor[2]. Bar indicates the region of the synthetic peptides used to prepare the polyclonal antibody.

4. Immunostaining (ABC method)

Immunohistochemical staining using the anti-EGF receptor polyclonal antibody was performed using an alternate protocol of Shiota *et al.*[16]. Deparaffinized sections of liver specimens which had been treated with 3% normal goat serum in order to block non-specific reactions were treated consecutively at room temperature with (1) the first antibody: anti-EGF receptor antibody (diluted 1 : 1000) overnight (2) the second antibody: 0.5% anti-rabbit IgG biotinylated goat antibody for 30 min, and (3) avidin DH-biotinylated horseradish peroxydase complex for 30 min. Reactions with (2) and (3) were performed using Vectastain® ABC Kit (Funakoshi Co. Tokyo).

Peroxydase staining was performed for 2–3 min using a solution of 10 mg 3,3′-diaminobenzidine-tetrahydrochloride in 100 ml of Tris-HCl (0.1 M pH 7.2) containing 0.02% hydrogenperoxide. Sections were counterstained for nuclei with 1% methyl green dissolved in veronal acetate buffer at pH 4.2.

5. Specificity of the anti-EGF receptor antibody

Specificity of the reaction was determined by the following three methods.

(1) Non-immune normal rabbit serum was used in place of the first antibody.

(2) The presence of EGF receptor has been reported in the smooth muscle layer in the human uterus[17–18]. Therefore, this antibody was examined for immunoreactivity in the rat uterus as a positive control.

(3) This antibody (diluted 1 : 800) was reacted with the above-mentioned excess synthetic peptides at 4°C for 24 hrs and centrifuged, and the supernatant was examined for immunoreactivity in the rat uterus (absorption test).

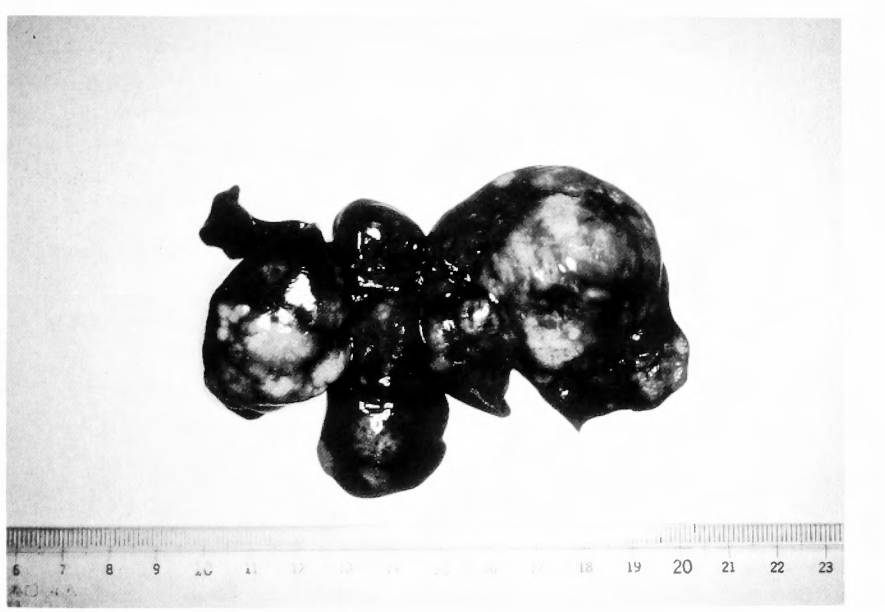

Fig. 2 Macroscopic findings of cholangiocarcinoma induced by 3′-Me-DAB. Multiple nodules of cholangiocarcinoma and hepatomegaly are observed.

Results

1. Macroscopic and microscopic findings in the liver tumor group

In 8 weeks after starting feeding with the 3′-Me-DAB diet, the animals showed remarkable hepatomegaly and diffuse multiple nodules were found macroscopically (Fig. 2). Histopathological examination showed cholangiocarcinoma nodules which were circumscribed with non-cancerous tissue but not encapsulated. The adjacent non-cancerous tissue consisted of hepatocytes which were irregularly arranged and had piknotic nuclei. These findings were suggestive of hepatic damage (Fig. 3a), whereas part of the surrounding non-cancerous liver tissue showed pseudocholangiole structure and regenerated capillaries, indicating hepatic regeneration. In 10 and 12 weeks after starting feeding with the 3′-Me-DAB diet, almost the same findings were obtained as were found in 8 weeks.

In 14 weeks after the initiation of feeding with the 3′-Me-DAB diet, nodules composed of atypical hepatocytes were encountered in addition to nodules of cholangiocarcinoma. The hepatocytes in such nodules contained large nuclei with multiple nucleoli and eosinophilic cytoplasm (Fig. 4a).

2. Expression of EGF receptor in the control group

In the normal rat liver, immunoreactive products against EGF receptor (hereafter referred to as immunoproducts) were diffusely distributed, although immunoproducts were only weakly positive. The immunoproducts were positive in the cytoplasm of hepatocytes. The localization of the EGF receptor immunoproducts showed a granular pattern (Fig. 5). The immunoreactivity was almost the same among 8, 10, 12, and 14 weeks.

3. Expression and localization of EGF receptor in the liver tumor group

Immunoproducts were positive both in the cholangiocarcinoma nodule and in the nodule of atypical hepatocytes. The immunoreactivity in these tissues was weak and similar to that observed in the control group. The immunoproducts were significantly more intensely positive in the non-cancerous liver tissue adjacent to the tumorous nodules than in the cancerous liver tissues of the carcinogen-treated animal (Fig. 3b, 4b). In the liver tissue distant from the tumorous nodules, however, immunoproducts were only weakly positive, and the immunoreactivity was comparable to that shown in the control group.

4. Specificity of anti-EGF receptor antibody

(1) No immunoproducts were found in the sections stained with non-immune normal rabbit serum.

(2) Immunoproducts were distinctively positive only in the smooth muscle layer of the rat uterus (Fig. 6), which was consistent with the reports on the human uterus[17-18].

(3) Immunoproducts in the absorption test were not detected in the rat uterus, indicating that the antibody was completely absorbed by the excess synthetic peptides.

Discussion

Damjanov *et al.*[12] studied immunohistochemical localization of EGF receptor in normal human tissues, and demonstrated that EGF receptor was expressed abundantly in the skin, mammary glands, pancreatic acini, the epithelia of the pancreatic duct, and hepatocytes. They classified the patterns of EGF receptor staining into two types, namely, linear cell surface pattern and cytoplasmic

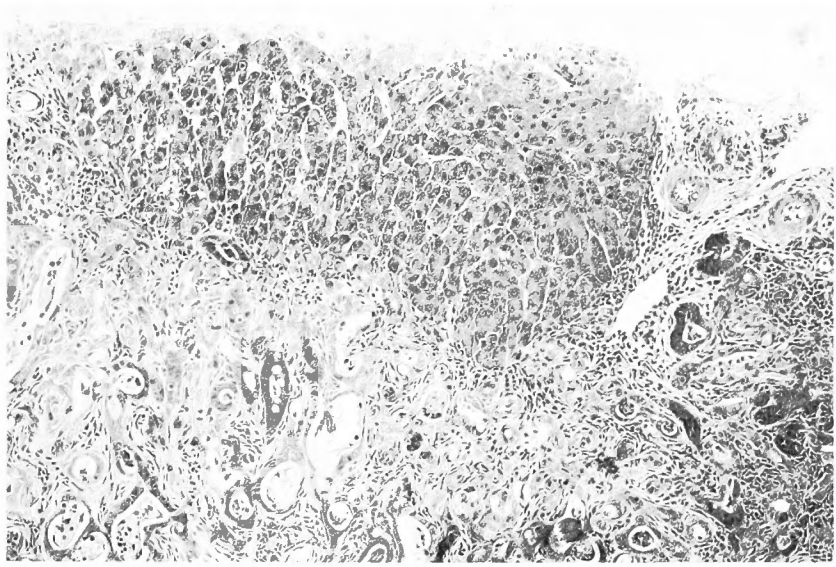

Fig. 3a Rat liver 8 weeks after feeding with 3′-Me-DAB (H.E. × 100). Note the non-cancerous tissue is observed adjacent to a nodule of cholangiocarcinoma. Irregular arrangement of hepatic cord and piknotic nuclei of hepatocytes suggest hepatic damage.

Fig. 3b The same tissue as Fig. 3a. EGF receptor of the rat liver 8 weeks after feeding with 3′-Me-DAB (× 100). Immunoproducts against EGF receptor are found in the nodule of cholangiocarcinoma, and much more intensely positive in damaged hepatocytes adjacent to the nodule.

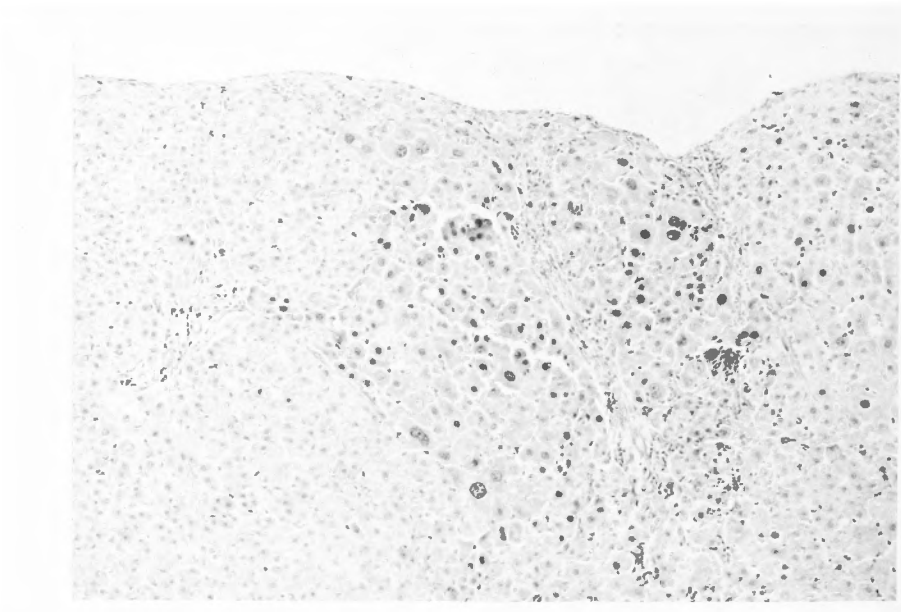

Fig. 4a Rat liver 14 weeks after feeding with 3′-Me-DAB (H.E. × 100). A nodule is composed of atypical hepatocytes which contain large nuclei with multiple nucleoli and eosinophilic cytoplasm.

Fig. 4b The same tissue as Fig. 4a. EGF receptor of the rat liver 14 weeks after feeding with 3′-Me-DAB (× 100). Immunoproducts against EGF receptor are intensely positive in the cytoplasm of hepatocytes surrounding the atypical nodule, and also positive in the atypical nodule.

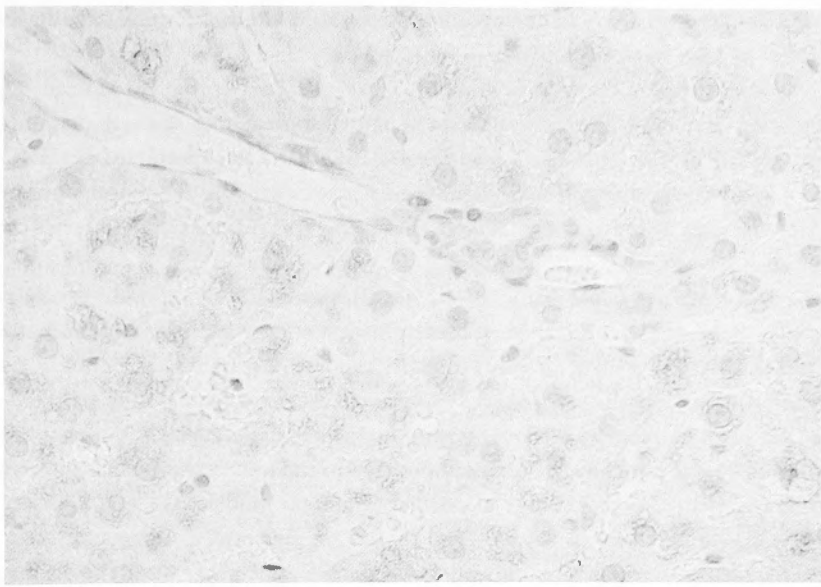

Fig. 5 EGF receptor of normal rat liver (×400). Immunoproducts against EGF receptor are positive in the cytoplasm of hepatocytes.

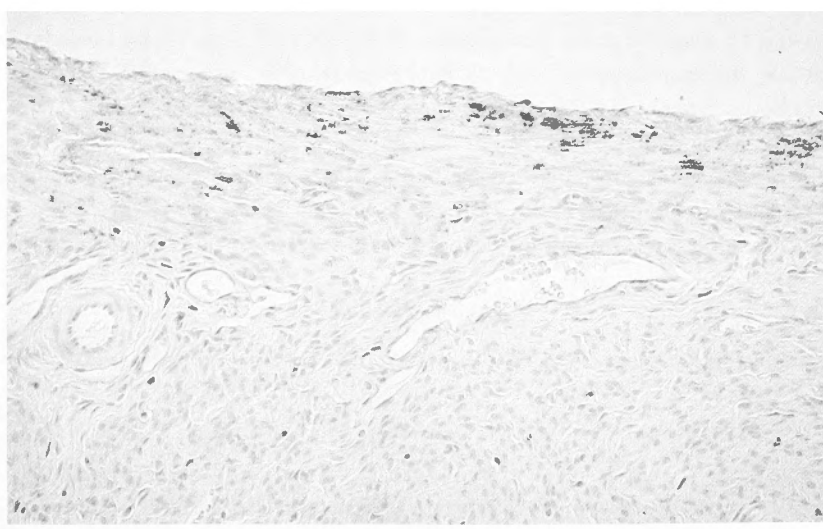

Fig. 6 Immunoproducts against EGF receptor are distinctively positive in the smooth muscle layer of the rat uterus (×200), indicating that the antibody specifically recognizes EGF receptor.

granular pattern. They showed that EGF receptor staining was of granular pattern in the cytoplasm of hepatocytes. The present study confirmed that the immunoproducts against EGF receptor were found in granular pattern in the cytoplasm of hepatocytes.

Absorption test revealed that the antibody used in our study recognized part of the autophosphorylation site of EGF receptor, and positive control test showed that this antibody specifically recognized EGF receptor. These results confirmed the specificity of this antibody to EGF receptor. In addition, this antibody did not crossreact with *v-erb B* oncogene products, which supports the fact that *v-erb B* products do not have the amino acid residues of EGF receptor which this antibody recognizes (see Fig. 1).

There are many reports concerning the overexpression of EGF receptor in various human cancer tissues[6–11]. Some correlation between the expression of EGF receptor and mechanisms of carcinogenesis has been proposed since Downward *et al.*[4] reported close similarity of EGF receptor and *v-erb B* oncogene products. In the present study, immunoreactivity against EGF receptor was positive both in cholangiocarcinoma nodules and atypical nodules of hepatocytes. It was noteworthy that the immunoproducts were much more intensely positive in the non-cancerous liver tissues adjacent to the tumorous regions. These findings in our experimental model were not consistent with the previous reports on human cancer tissues[9–11]. In the present study, some interaction between tumorous nodules and the surrounding non-cancerous tissues may have caused the overexpression of EGF receptor. Since the adjacent non-cancerous liver tissues showed histological signs of hepatic damage and liver regeneration (Fig. 3a, 4a), it is possible that hepatocytes damaged by 3′-Me-DAB produced EGF receptor during the regenerating process. In addition, our recent study on the pancreas of streptozotocin (STZ)-induced diabetic rats indicated that EGF receptor was strongly expressed in the acini around degenerated islets (unpublished observations). In this regard, it seems probable that EGF receptor is expressed in the tissues around damaged lesions. Further studies are needed to investigate the roles of EGF receptor in the process of regeneration of the liver.

The present study also suggests that immunohistochemistry seems to offer special interests for investigating EGF receptor expression in the liver. Immunohistochemistry has a great merit that these findings can be observed in an intact tissue, which is hardly possible by biochemical technique. In the future, the significance of EGF receptor expression in regeneration and other pathologic conditions will be further clarified by studies using various experimental models.

Part of this study was presented at the 3rd World Congress of Hepato-Pancreato-Biliary Surgery in London, June 8, 1990, and nominated as an excellent study in the poster session.

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和文抄録

肝臓における上皮細胞増殖因子 (EGF) 受容体に関する免疫組織化学的研究

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特異的な抗 EGF 受容体抗体を用いた免疫組織化学的手法で，ラットの肝臓を用いて EGF 受容体の発現を検討した．対照群（正常肝）における EGF 受容体の免疫組織染色では，肝細胞の細胞質にその反応産物が均一に認められた．

実験群（肝腫瘍群）には，3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) の投与により，8週目には胆管癌結節が，14週目には胆管癌結節と異型肝細胞の結節が観察された．肝腫瘍群の肝臓においては，胆管癌を示す組織内や異型肝細胞の結節内においても一部で反応産物が観察された．しかしながら，これらの結節に隣接する非癌部の肝組織においては，肝細胞の細胞質に EGF 受容体を示す反応産物が，対照群の肝細胞や胆管癌及び異型肝細胞の組織における染色性に比べ明らかに強く染色される領域が認められた．本研究におけるこれらの興味深い現象から，発癌に際し癌組織と非癌部組織との間の相互作用の結果，EGF 受容体が過剰発現する可能性，あるいは 3′-Me-DAB による肝障害に伴い肝再生の転機とともに EGF 受容体が過剰発現する可能性が示唆された．